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Basal Phosphorylation of the PEST Domain in IκBβ Regulates Its Functional Interaction with the c-*rel* Proto-Oncogene Product

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The product of the c-*rel* proto-oncogene (c-Rel) belongs to the NF-κB/Rel family of polypeptides and has been implicated in the transcriptional control of cell proliferation and immune function. In human T lymphocytes, c-Rel is sequestered in the cytoplasmic compartment by constitutively phosphorylated inhibitors, including IκBα and IκBβ. Studies with bacterially expressed forms of these inhibitory proteins revealed that unphosphorylated IκBα but not IκBβ assembles with c-Rel and inhibits its DNA binding activity. Furthermore, latent IκBβ–c-Rel complexes derived from mammalian cells were sensitive to phosphatase treatment, whereas IκBα–c-Rel complexes were resistant. We have identified a constitutive protein kinase in unstimulated T cells that associates with and phosphorylates IκBβ in vitro. The substrate specificity, electrophoretic mobility, and antigenic properties of this IκBβ-associated kinase (BAK) suggest identity with casein kinase II (CKII), an enzyme known to mediate basal phosphorylation of IκBα. Phosphorylation of recombinant IκBβ by either BAK or CKII restored the capacity of this inhibitor to antagonize the DNA binding activity of c-Rel. Peptide mapping and mutational analyses localized the bulk of the basal phosphorylation sites in IκBβ to the C-terminal PEST domain, which contains two potential acceptors for CKII-mediated phosphoryl group transfer (Ser-313 and Ser-315). Point mutations introduced into the full-length inhibitor at Ser-313 and Ser-315 led to a significant reduction in the phosphorylation of IκBβ and severely impaired its c-Rel inhibitory function in vivo. Taken together, these findings strongly suggest that basal phosphorylation of the PEST domain of IκBβ at consensus CKII sites is required for the efficient formation of latent IκBβ–c-Rel complexes.

The NF-κB/Rel family of transcription factors plays a critical role in the development of a normal immune response (39, 65, 75). The nuclear activities of NF-κB/Rel proteins are regulated from the cytoplasmic compartment by interactions with a set of inhibitory proteins, including IκBα and IκBβ (26, 71, 74). Cellular stimulation leads to the rapid degradation of these conditionally labile inhibitors and the transit of active NF-κB/Rel complexes to the nucleus (9, 26, 74). Primary structural and functional analyses have indicated that IκBα and IκBβ are organized as tripartite molecules containing (i) an N-terminal regulatory domain required for induced proteolytic breakdown (15, 16, 18, 21, 49, 62, 68, 73, 76), (ii) a central Rel-interactive domain composed of six ankyrin repeat motifs (25, 33, 35), and (iii) a highly acidic C-terminal region which is rich in proline, glutamic acid, serine, and threonine residues (30, 72). Sequences of the latter type, termed PEST domains, have been implicated as *cis*-acting proteolytic signals that determine the metabolic turnover rates of specific proteins (56, 57). However, the function of the PEST domains in IκB proteins remains controversial. In contrast to prior reports (16, 76), recent mutational studies have suggested that the PEST domain of IκBα is dispensable for its signal-induced turnover in activated cells (68). Other experiments have indicated that phosphorylation of the IκBα PEST domain by casein kinase II regulates basal rather than induced turnover of this inhibitor (46, 48, 64). Furthermore, the structurally related IκBβ protein is resistant to proteolytic breakdown in response to a subset of agents that lead to the inactivation of IκBα, thus suggesting that these PEST-containing inhibitors are differentially regulated (71, 72). However, a definite role for either basal or inducible phosphorylation in IκBβ function has not been established (4).

The prototypical form of NF-κB contains p50 (NF-κB1) and a transactivating subunit, termed RelA (p65) (29, 66). The p50 subunit can also form complexes with a structurally related transactivating subunit encoded by the c-*rel* proto-oncogene (6, 23, 38). Prior reports have provided in vivo evidence for the existence of homodimeric forms of RelA and c-Rel, both of which bind consensus κB motifs in vitro (19, 27, 41, 52). Although the RelA transactivating subunit of NF-κB has been extensively characterized (29, 66), much less is known about the function and regulation of c-Rel. Depending on the recipient cell type, overexpression of c-Rel can lead to either neoplastic transformation or programmed cell death (1). Recent gene targeting studies indicate that c-Rel plays a critical role in the regulation of antigen-directed lymphocyte proliferation and immune function (39). Consistent with this, c-Rel has been implicated in the transcriptional control of cellular and viral genes that are induced during T-cell activation, including those encoding the growth factor interleukin-2 (28, 39), the alpha subunit of the high-affinity interleukin-2 receptor (70), and human immunodeficiency virus type 1 (HIV-1) proteins (23). Furthermore, emerging evidence suggests that latent cytoplasmic complexes containing c-Rel are targets for signals discharged from cell surface CD28, which serves as a costimulatory receptor in the activation of T-cell immune responses (17, 28). In particular, these studies revealed that CD28 signaling

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leads to the sustained downregulation of IκB function, which presumably potentiates the translocation of c-Rel-containing complexes to the nuclear compartment (42, 43).

In the present study, we have explored the mechanism by which IκBβ sequesters c-Rel in the cytoplasmic compartment. We have found that the C-terminal PEST domain of IκBβ is basally phosphorylated in human T lymphocytes. Recombinant unphosphorylated forms of IκBβ inhibit the DNA binding activity of RelA but not that of c-Rel. However, phosphorylation of the C-terminal PEST domain of IκBβ rescues its c-Rel inhibitory function in vitro. Several biochemical lines of evidence reveal that the serine/threonine protein kinase casein kinase II (CKII), or a closely related kinase, is responsible for the phosphorylation of the IκBβ PEST domain. In addition, we have genetically mapped the functionally relevant phosphoacceptor sites in the PEST domain of IκBβ to Ser-313 and Ser-315, both of which reside within consensus CKII recognition motifs. Taken together, these findings suggest that CKII-mediated phosphorylation of the PEST domain present in IκBβ is a prerequisite for the functional interaction of this cytoplasmic inhibitor with c-Rel.

MATERIALS AND METHODS

Purification of recombinant IκB. Full-length cDNAs for human IκBα (30) and IκBβ (45) (provided by D. Moore) were modified by PCR-assisted amplification with oligonucleotide primers that fused epitope tags in frame with their N-terminal (FLAG tag) and C-terminal (HIS tag) coding sequences (11, 36, 55). Alternatively, for some experiments, IκBβ cDNAs that selectively expressed the N-terminal FLAG tag were generated. To facilitate radiolabeling in vitro, the N-terminal FLAG epitope was engineered to contain a phosphorylation site for the catalytic subunit of heart muscle kinase (HMK) (11). PCR fragments were inserted into the polylinker of either the prokaryotic expression vector pET-24(a) (Novagen) or the eukaryotic expression vector pCMV4 (3). Codon substitutions were introduced into these IκB constructs with a Muta-Gene mutagenesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The derivative of IκBα containing alanine replacements at Ser-283, Ser-288, Thr-291, Ser-293, Thr-296, and Thr-299 (mutant K [25]) was provided by Nancy Rice. Molecularly tagged IκB constructs in pET-24(a) were transformed into *Escherichia coli* BL21(DE3) cells. The transformants were grown to early log phase in Luria-Bertani medium, treated with 1 mM isopropyl-β-D-thiogalactoside for 3 h, and harvested by centrifugation. These cells were resuspended in HB buffer (25 mM Tris [pH 7.9], 0.5 M NaCl, 5 mM imidazole, 0.1% Nonidet P-40) and disrupted by sonication. Recombinant proteins were purified from clarified lysates by adsorption to a nickel chelate agarose resin (His-Bind; Novagen) and elution with HB buffer containing 500 mM imidazole. The purified proteins were desalted with Centricon-30 microconcentrators (Amicon) and stored at −80°C in 20 mM Tris (pH 7.5)–100 mM NaCl–1 mM dithiothreitol.

Transient transfections. Jurkat T lymphocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Approximately 10^7 cells were transfected by electroporation as described previously (15) with the indicated Rel and FLAG-tagged IκB expression vectors (23, 30, 45, 58). Alternatively, plasmids were introduced into A293T cells maintained in DMEM (24) (provided by M. Malim) by published calcium phosphate transfection procedures (20). The chloramphenicol acetyltransferase (CAT) reporter plasmid containing two NF-κB binding sites from the HIV-1 enhancer linked to a heterologous TATA box (HIV-κB-CAT) has been described previously (67). For CAT assays, whole-cell extracts were prepared from transfectants, normalized for protein concentration (14), and analyzed by a diffusion-based liquid scintillation counting method (53).

Subcellular fractionation and binding assays. Whole cell extracts were prepared by lysis with ELB buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.0], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol) supplemented with a previously described cocktail of protease inhibitors (7). Cytoplasmic and nuclear extracts were prepared by the method of Schreiber et al. (63). Gel mobility shift assays were performed with a ^{32}P-radiolabeled duplex (κB-pd) derived from κB enhancer sequences in the interleukin-2 receptor α subunit promoter (5′-CAACGGCAGGGGAATTCCC CTCTCCTT-3′) (7). Photoreactive derivatives of the radiolabeled κB-pd probe containing 5-bromo-2′-deoxyuridine 5′-triphosphate were synthesized as described previously (5). DNA binding reactions (20 μl) contained 5 μg of protein extract, 5 fmol of radiolabeled probe, 2 μg of double-stranded poly(dI-dC), and 10 μg of bovine serum albumin buffered in a solution containing 20 mM HEPES (pH 7.9), 125 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5% glycerol, and 1% Nonidet P-40. In some experiments, protein extracts were treated with calf intestinal phosphatase (CIP) prior to mobility shift analyses. Dephosphorylation reactions were performed at 30°C for 1 h in a solution containing 25 mM HEPES (pH 7.9), 0.1 mM EDTA, 5% glycerol, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 0.5 mM dithiothreitol. Treated samples were then equilibrated in the DNA binding reaction buffer described above. Resultant nucleoprotein complexes were resolved on native 5% polyacrylamide gels and detected by autoradiography (12).

For DNA-protein cross-linking, reaction mixtures were irradiated at 300 nm for 30 min with a Fotodyne UV transilluminator. Adducts were either analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or fractionated with subunit-specific antisera prior to electrophoresis (7). For protein shift assays, purified recombinant IκBα and IκBβ were radiolabeled at the N-terminal FLAG epitope with bovine HMK (Sigma) and [γ-^{32}P]ATP as described previously (11). Radiolabeled inhibitors (~10,000 cpm; 100 pg) were incubated for 15 min at 23°C with whole-cell extracts (5 μg) prepared from either c-Rel-, RelA-, or p50-transfected A293T cells. Resultant IκB-Rel complexes were resolved on 5% polyacrylamide gels under nondenaturing conditions and visualized by autoradiography (12).

In vitro phosphorylation and in-gel kinase assays. Whole-cell extracts were prepared from Jurkat T lymphocytes by lysis with HB buffer. Clarified protein lysates (10 mg in 1 ml of HB buffer) were incubated with purified recombinant IκBβ (10 μg) in the presence of nickel chelate agarose beads (packed volume, 100 μl) for 2 to 4 h at 4°C. The resin was washed three times with HB buffer, and IκBβ-associated kinases (BAK) were eluted with HB buffer containing 6 M urea. Unless indicated otherwise, in vitro kinase reactions (20 μl) were performed in KN buffer (25 mM Tris-Cl [pH 7.6], 100 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$) containing 25 μM ATP, 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), 20 to 50 ng of purified recombinant IκBβ, and 1 μl of undialyzed BAK. Control reactions contained 5 U of purified CKII (Promega) or bovine HMK. Reactions were incubated at 30°C for 30 min and terminated by the addition of 2% SDS. Phosphorylated products were resolved by SDS-PAGE and visualized by autoradiography.

For in-gel kinase assays (32), BAK (10 μl) or CKII (5 U) was loaded on 10% polyacrylamide–SDS gels polymerized in the presence of purified recombinant IκBβ (10 μg/ml). Following electrophoresis, the gels were washed sequentially with (i) 100 ml of 50 mM Tris (pH 7.6) containing 20% isopropanol, (ii) buffer A (50 mM Tris [pH 7.6], 5 mM β-mercaptoethanol), and (iii) buffer A containing 6 M urea. To renature the immobilized protein, the gels were sequentially incubated in buffer A solutions containing 0.05% Tween 20 and graded concentrations of urea (3, 1.5, and 0.75 M) and then stored overnight at 4°C in buffer A–0.05% Tween 20. Gels were equilibrated in 50 ml of KN buffer, incubated in 10 ml of KN buffer containing 50 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) for 1 h at 23°C, and washed extensively in 5% trichloroacetic acid and 1% sodium PP_i to remove unincorporated radioisotopes.

Immunoblotting and immunodepletion studies. Preparations of BAK (10 μl) were fractionated on 10% polyacrylamide–SDS gels and transferred to polyvinylidene difluoride (PVDF) membranes (DuPont). The membranes were blocked (15) and immunoblotted with peptide-specific antibodies for CKII (Upstate Biotechnology, Inc.). Immunoreactive polypeptides were detected by using donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase in an enhanced chemiluminescence system as specified by the manufacturer (Dupont NEN). Immunodepletion of CKII from BAK preparations was performed in KN buffer (50 μl) containing affinity-purified BAK (2 μl) and rabbit anti-CKII antibodies (12 μg). Immunoreactive complexes were removed by the addition of protein A-agarose beads (15 μl). Supernatants were supplemented with 25 μM ATP, 10 μCi of [γ-^{32}P]ATP, and purified IκBβ (20 ng) and then were assayed for IκBβ kinase activity as described above.

Analysis of phosphorylated IκB proteins. To analyze the phosphorylation status of endogenous IκB, Jurkat T lymphocytes (5×10^7) were metabolically radiolabeled for 4 h with $^{32}P_i$ (1 mCi/ml; ICN) in phosphate-depleted RPMI 1640 medium. Cytosolic extracts (63) were prepared in the presence of phosphatase inhibitors (49), equilibrated in ELB buffer, and immunoprecipitated with peptide-specific antisera raised against either RelA (amino acids 1 to 21) or c-Rel (amino acids 573 to 587). Immune complexes were washed with ELB buffer and then boiled for 10 min in the presence of 1% SDS. Denatured proteins were diluted 1:10 in ELB buffer and reprecipitated with antibodies specific for the C terminus of either human IκBα (amino acids 289 to 317) or IκBβ (C-20; Santa Cruz Biotechnology, Inc.). Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography.

For in vivo phosphorylation analysis of ectopic forms of IκBβ, Jurkat T cells (10^7) were transfected by electroporation (15) with pCMV4 expression vectors encoding FLAG-tagged derivatives of IκBβ (20 μg). Approximately 36 h post-transfection, cells were radiolabeled with $^{32}P_i$ as described above. Whole-cell extracts were prepared by lysis in ELB buffer containing a cocktail of protease (7) and phosphatase (49) inhibitors. Lysates were boiled in the presence of 1% SDS, diluted 1:10 in ELB buffer, and then incubated with 20 μl of agarose beads conjugated to monoclonal anti-FLAG antibodies (M2; IBI-Kodak). Immunoprecipitates were washed five times at high stringency with RIPA buffer (7) and analyzed by SDS-PAGE as described above.

Peptide mapping and phosphoamino acid analysis. Purified IκBβ isolated from *E. coli* was radiolabeled with [γ-^{32}P]ATP and either CKII or BAK as described above. Alternatively, A293T cells transfected with FLAG- and HIS-tagged IκBβ expression vectors were cultured in the presence of $^{32}P_i$ (1 mCi/ml) for 4 h at 37°C and whole-cell extracts were prepared in ELB lysis buffer. In

either case, radiolabeled IκBβ was immunoprecipitated with peptide-specific antibodies (C-20) and protein A-agarose, eluted from the beads by boiling, and coupled to anti-FLAG antibody beads (20 μl). Solid-phase proteins were equilibrated with 25 mM Tris (pH 8.5)–1 mM EDTA and digested with 500 ng of endoproteinase Lys-C (Boehringer Mannheim) at room temperature for 14 h. Cleavage products were fractionated with either an antiserum raised against the N terminus of IκBβ (G-20; Santa Cruz Biotechnology, Inc.) or a nickel chelate resin which binds the C-terminal HIS tag of IκBβ. Proteolytic fragments were analyzed on high-resolution Tris-Tricine gels as described previously (61). For phosphoamino acid analysis, radiolabeled IκBβ was resolved by SDS-PAGE, transferred to PVDF membranes, and hydrolyzed in 6 M hydrochloric acid (1 h; 110°C). Hydrolysates were separated by two-dimensional electrophoresis as described previously (pH 1.9 followed by pH 3.5) (13). Phosphoamino acids were identified on the basis of their electrophoretic mobilities relative to those of standards stained with ninhydrin (Sigma).

RESULTS

Association of c-Rel with phosphorylated IκBα and IκBβ. Prior studies have shown that c-Rel and RelA are predominantly located in the cytoplasmic compartment of resting T lymphocytes because of their physical association with IκB proteins (9, 74). To determine the IκB composition of latent complexes containing these transactivating subunits, coimmunoprecipitation studies with IκB-specific antisera and cytoplasmic extracts from either primary or transformed human T lymphocytes were performed. As shown in Fig. 1A, c-Rel and RelA were readily detected in latent cytoplasmic complexes containing IκBα (lanes 1 and 3). Regardless of the extract source, a similar pattern of Rel partitioning was observed in experiments conducted with an IκBβ-specific antiserum (lanes 2 and 4). These data firmly establish that both primary and transformed T cells express cytoplasmic complexes containing c-Rel and IκBβ.

To evaluate the phosphorylation status of IκBβ in these sequestered complexes, unstimulated Jurkat T cells were metabolically radiolabeled with $^{32}P_i$ and latent Rel complexes were isolated from cytoplasmic extracts with peptide antisera for either RelA or c-Rel. Immune complexes were disrupted by boiling in the presence of SDS, immunoprecipitated with anti-IκB antisera, and analyzed by SDS-PAGE. Consistent with prior studies using other cell types (8, 22), these metabolic labeling experiments revealed that the fraction of cytoplasmic IκBα associated with either RelA or c-Rel is basally phosphorylated (Fig. 1B, lanes 1 and 3). More importantly, we found that Rel-bound IκBβ is also basally phosphorylated in human T cells (Fig. 1B, lanes 2 and 4). Although the overall stoichiometry of phosphorylation remains uncertain, the quantitative differences in radiolabeled IκB associated with RelA and c-Rel correlated with the relative abundance of these transactivating subunits in cytoplasmic extracts (see Fig. 1A). Taken together, these subunit compositional analyses demonstrate that c-Rel is stably associated with phosphorylated IκBβ in the cytoplasm of unstimulated T lymphocytes.

Bacterial IκBα but not IκBβ inhibits the formation of c-Rel–DNA complexes. To explore the functional consequences of basal IκB phosphorylation, unmodified forms of IκBα and IκBβ were produced as recombinant proteins (rIκBα and rIκBβ, respectively) in *E. coli*. Alternatively, in order to produce basally phosphorylated forms of these inhibitors (pIκBα and pIκBβ, respectively), the corresponding cDNAs were overexpressed in transfected A293T cells. As shown in Fig. 2A, pIκBβ and rIκBβ (lanes 3 and 4) migrated with distinct electrophoretic mobilities during SDS-PAGE, presumably reflecting their different phosphorylation states. From A293T transfectants, we prepared protein extracts containing either c-Rel or RelA homodimers, both of which have been detected in untransfected cells (19, 27, 41, 52). DNA-protein cross-linking studies conducted with these extracts revealed photoreactive

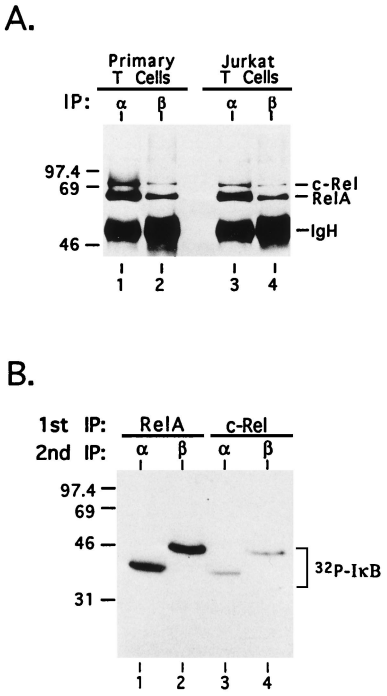


FIG. 1. Phosphorylated IκBβ forms cytoplasmic complexes with c-Rel. (A) Association of IκBβ with c-Rel in primary and transformed T cells. Human peripheral blood lymphocytes were purified from buffy-coat cells by sheep erythrocyte rosetting as described previously (60). Cytoplasmic extracts from the indicated cells were subjected to immunoprecipitation (IP) with antisera specific for either IκBα (lanes 1 and 3) or IκBβ (lanes 2 and 4). Immune complexes were fractionated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with a combination of c-Rel- and RelA-specific antisera. Molecular mass standards are given in kilodaltons. The position of the immunoglobulin heavy chain (IgH) detected with secondary antibody is indicated. (B) c-Rel-bound IκBβ is basally phosphorylated. Jurkat T cells were metabolically radiolabeled with $^{32}P_i$, and cytoplasmic extracts were prepared. Latent Rel-IκB complexes were initially fractionated by immunoprecipitation with peptide antisera specific for either RelA (lanes 1 and 2) or c-Rel (lanes 3 and 4). Affinity-purified complexes were heat denatured, subjected to a second round of immunoprecipitation with the indicated IκB-specific antiserum, and resolved by SDS-PAGE. Phosphoproteins were visualized by autoradiography.

adducts corresponding in sizes to those predicted for either c-Rel or RelA (Fig. 2B, lanes 2 and 3, respectively) when covalently bound to a 27-bp κB probe (50). Using these extracts, mixing experiments were performed with graded amounts of IκB protein. As shown in Fig. 2C, both pIκBα and pIκBβ potently inhibited the DNA binding of these Rel protein dimers in gel retardation assays (lanes 2 to 5). However, in contrast to rIκBα, rIκBβ failed to sequester the DNA binding activity of c-Rel homodimers (lanes 7 to 10).

Reciprocal mixing experiments conducted with rIκBβ and full-length RelA homodimers revealed dose-dependent inhibition (bottom panel, lanes 7 to 10). However, when these DNA binding reaction mixtures were supplemented with high concentrations of rIκBβ a minor supershifted nucleoprotein complex was generated. This nucleoprotein complex was not detected in titrations performed with pIκBβ (bottom panel, lanes 2 to 5). Compositional analysis of this minor product revealed the presence of rIκBβ (data not shown), thus demonstrating that unmodified IκBβ can engage DNA indirectly in vitro when tethered to RelA. Although the physiologic relevance of this unexpected finding remains to be determined, the data shown in Fig. 1 and 2 strongly suggest that the phosphorylation status of IκBβ plays a critical role in the regulation of c-Rel DNA binding activity.

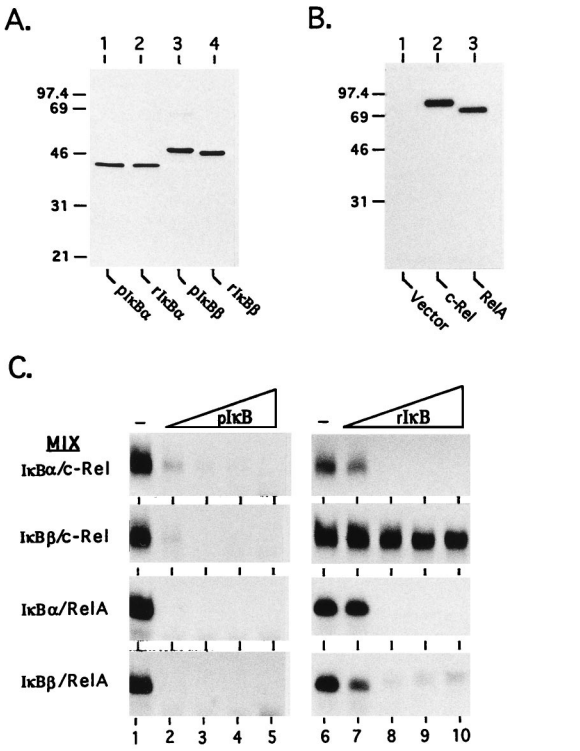


FIG. 2. Unmodified IκBβ lacks c-Rel inhibitory activity. (A) Electrophoretic mobilities of phosphorylated (pIκB) and unmodified (rIκB) inhibitors. Tagged IκBα and IκBβ proteins were overexpressed in A293T cells (lanes 1 and 3) or in *E. coli* (lanes 2 and 4). Bacterially expressed proteins (10 ng) purified by nickel chelate affinity chromatography and A293T whole-cell extracts (5 μg) were fractionated by SDS-PAGE and immunoblotted with anti-FLAG M2 antibody. Molecular mass standards are given in kilodaltons. (B) DNA binding analysis of Rel-transfected cells. Whole-cell extracts were prepared from A293T cells transfected with pCMV4 expression vectors (7.5 μg) encoding either c-Rel (lane 2) or RelA (lane 3). Extracts (5 μg) were incubated with a photoreactive ^{32}P-labeled κB probe, irradiated with UV light, and fractionated by SDS-PAGE. Cross-linked nucleoprotein complexes were detected by autoradiography. Reaction mixtures containing control extracts from A293T cells transfected with empty pCMV4 vector were analyzed in lane 1. (C) Inhibition of Rel DNA binding by phosphorylated (pIκB) and unmodified (rIκB) inhibitors. A293T extracts (5 μg) containing homodimers of either c-Rel (top two panels) or RelA (bottom two panels) were mixed with graded amounts of either A293T extracts containing overexpressed pIκB (5, 10, 20, and 40 μg) or bacterially expressed rIκB (0.5, 5, 50, and 100 ng). Reaction mixtures were incubated with radiolabeled κB probe under standard reaction conditions and analyzed on 5% polyacrylamide gels. Control reaction mixtures lacking IκB were analyzed in lanes 1 and 6. Only the major nucleoprotein complexes detected by autoradiography are shown in this composite.

Unmodified IκBβ is defective in c-Rel binding activity. Recent studies have suggested that the PEST domain of IκBα is required for inhibition of DNA binding but is dispensable for association of this inhibitor with c-Rel (25, 59). To determine whether rIκBα and rIκBβ have the capacity to physically engage c-Rel, their N-terminal epitope tags were radiolabeled with [γ-^{32}P]ATP and HMK. These ^{32}P-labeled inhibitors were mixed with in vivo-synthesized RelA or c-Rel (Fig. 2B), and the resultant Rel-IκB complexes were resolved on 5% polyacrylamide gels under nondenaturing conditions. As shown in Fig. 3, addition of either c-Rel or RelA severely retarded the electrophoretic mobility of radiolabeled IκBα compared with that in Rel-deficient binding reactions (lanes 1, 2, and 4). In keeping with the functional data (Fig. 2C), RelA also formed stable complexes with radiolabeled rIκBβ (Fig. 3, lanes 7 and 8). In sharp contrast, interaction of rIκBβ with c-Rel in these protein shift assays was not detected (lane 10). This finding is fully consistent with the inability of IκBβ to block c-Rel DNA binding activity when produced as an unmodified inhibitor in bacteria (Fig. 2C). We conclude that IκBβ must be posttranslationally modified in eukaryotic cells to acquire its c-Rel targeting function.

Dephosphorylation of cytoplasmic IκBβ–c-Rel complexes activates c-Rel. Prior studies have shown that the exposure of IκBβ to alkaline phosphatase abolishes its ability to inhibit the DNA binding activity of NF-κB (37, 47). Coupled with the finding that c-Rel-bound IκBβ is basally phosphorylated in human T cells (Fig. 1B), we considered the possibility that the formation of c-Rel–IκBβ complexes might be dependent on the phosphorylation status of IκBβ. To explore this possibility, we first monitored the sensitivity of IκBβ–c-Rel complexes to CIP. For these experiments, latent complexes were generated by mixing extracts from A293T cells transfected independently with expression vectors for either c-Rel, RelA, IκBα, or IκBβ. Following treatment with CIP, these complexes were cross-linked to ^{32}P-labeled κB oligonucleotides containing 5-bromo-2′-deoxyuridine 5′-triphosphate and analyzed by SDS-PAGE (Fig. 4). In IκB-deficient controls, photoreactive adducts containing c-Rel were readily detected (top panel, lane 1). The DNA binding activity of c-Rel homodimers was significantly reduced in reaction mixtures containing either pIκBα (lane 2) or pIκBβ (lane 5). Treatment of IκBβ–c-Rel complexes with CIP led to the full recovery of c-Rel DNA binding activity (lane 6), whereas IκBα–c-Rel complexes were resistant (lane 3). Experiments conducted with bacterially expressed c-Rel confirmed that this recombinant protein can engage pIκBβ (data not shown), thus suggesting that IκBβ rather than c-Rel is inactivated by CIP. Furthermore, phosphatase treatment was associated with a subtle but reproducible increase in the electrophoretic mobility of IκBβ during SDS-PAGE (Fig. 4, bottom panel, lane 6). This alteration was not detected in parallel studies with IκBα (lane 3). In contrast to that of c-Rel, the functional integrity of latent complexes containing RelA homodimers was unaffected by phosphatase treatment (Fig. 4,

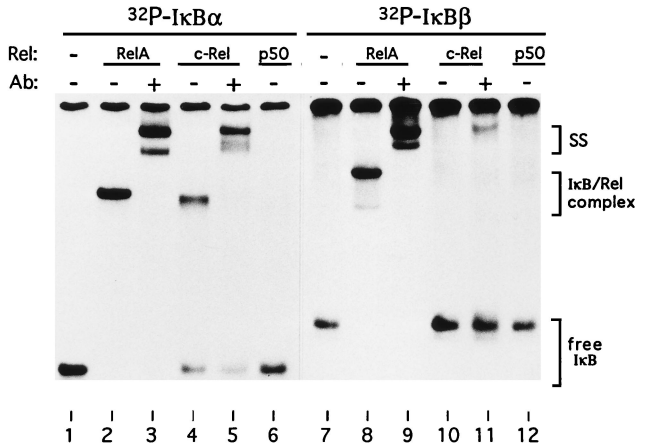


FIG. 3. Bacterially expressed IκBα but not IκBβ forms stable complexes with c-Rel. Affinity-purified IκBα and IκBβ from *E. coli* were ^{32}P-labeled with HMK. The indicated radiolabeled inhibitor (10^4 cpm; 100 pg) was incubated with lysates (5 μg) from A293T cells transfected with expression vectors for either RelA (lanes 2, 3, 8, and 9), c-Rel (lanes 4, 5, 10, and 11), or p50 (lanes 6 and 12). Where indicated (+), binding reactions were supplemented with the appropriate Rel subunit-specific antiserum (Ab). Resultant Rel-IκB complexes were resolved on a native 5% polyacrylamide gel and detected by autoradiography. The positions of free IκB, IκB-Rel complexes, and antibody-supershifted (SS) species are indicated.

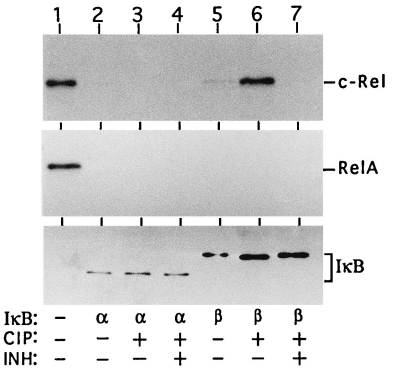


FIG. 4. Latent IκBβ–c-Rel complexes are sensitive to phosphatase treatment. Mammalian A293T cells were independently transfected with pCMV4 expression vectors (7.5 μg) encoding either c-Rel, RelA, IκBα, or IκBβ. Whole-cell extracts (5 μg) from recipient cells expressing either c-Rel or RelA were incubated with A293T extracts containing the indicated form of IκB (10 μg). Assembled IκB-Rel complexes (10 μl) were treated with calf intestinal phosphatase (7 U) for 1 h at 30°C. Control CIP reactions (lanes 4 and 7) were supplemented with a cocktail of phosphatase inhibitors (2.5 mM sodium PP_i, 250 μM sodium molybdate, 2.5 mM sodium orthovanadate, and 12.5 mM sodium fluoride) (INH). Reaction mixtures were incubated with radiolabeled κB-pd probe containing 5-bromo-2′-deoxyuridine 5′-triphosphate and cross-linked by exposure to UV light. Photoreactive adducts were immunoprecipitated with either c-Rel- or RelA-specific antisera (top and middle panel, respectively), resolved by SDS-PAGE, and visualized by autoradiography. To monitor IκB protein in CIP-treated samples, an aliquot (10%) from each c-Rel DNA binding reaction was subjected to SDS-PAGE and immunoblotting with monoclonal anti-FLAG M2 antibodies (bottommost panel). Similar results were obtained with RelA and IκB mixtures.

middle panel). Together, these findings provide additional evidence that the basal phosphorylation status of IκBβ influences its capacity to regulate the DNA binding activity of c-Rel but not RelA homodimers.

CKII is an IκBβ kinase. Recent biochemical studies have indicated that residues within the PEST domain of IκBα are basally phosphorylated by the serine/threonine kinase CKII (8, 46, 48, 64). Although unmodified IκBα and IκBβ possess striking differences in c-Rel-specific targeting (Fig. 2C and 3), both of these inhibitors contain C-terminal PEST sequences (30, 45, 72). To explore whether CKII regulates basal phosphorylation of IκBβ, we incubated whole-cell extracts from unstimulated Jurkat T lymphocytes with rIκBβ and nickel chelate agarose beads, eluted the bound proteins with urea, and analyzed their enzymatic activity by in-gel kinase assays (32). In gels polymerized with rIκBβ, two distinct BAK activities with relative molecular masses of approximately 40 and 45 kDa were detected (Fig. 5A, lane 2). The expression of these activities in T cells was unaffected by stimulation with phorbol 12-myristate 13-acetate and ionomycin, a combination which mimics activation through the T-cell receptor (data not shown). More importantly, these electrophoretically distinct kinases comigrated precisely with the α and α′ catalytic subunits of CKII on SDS gels (lane 1).

To further characterize these kinase activities, BAK preparations on immunoblots were probed with antibodies generated against CKII. These studies identified an immunoreactive 40-kDa protein (Fig. 5B, lane 2) which exhibited an electrophoretic mobility indistinguishable from that of the α subunit of CKII (lane 1). To extend these findings, we performed in vitro kinase assays using rIκBβ as an enzyme substrate in combination with either CKII or affinity-purified preparations of BAK from Jurkat T cells. Immunodepletion of CKII from BAK samples was associated with a significant loss of IκBβ kinase activity in these eluates (Fig. 5C). Consistent with the known properties of CKII (51), rIκBβ was efficiently phosphorylated by CKII and BAK in kinase reaction mixtures supplemented with GTP rather than ATP as the phosphate donor (Fig. 5D, lanes 3 and 6). Furthermore, phosphorylation of rIκBβ by either CKII or BAK was dramatically inhibited in the presence of heparin, a well-recognized antagonist of CKII enzymatic activity (lanes 2 and 5) (31). Taken together, these studies provide strong evidence that CKII is an IκBβ kinase which is either identical to or closely related to BAK.

Phosphorylation of rIκBβ rescues its c-Rel inhibitory function. Although these data clearly showed that CKII can phosphorylate IκBβ in vitro, the functional consequences of this modification remained unclear. To determine whether this enzymatic reaction influences IκBβ function, purified rIκBβ was

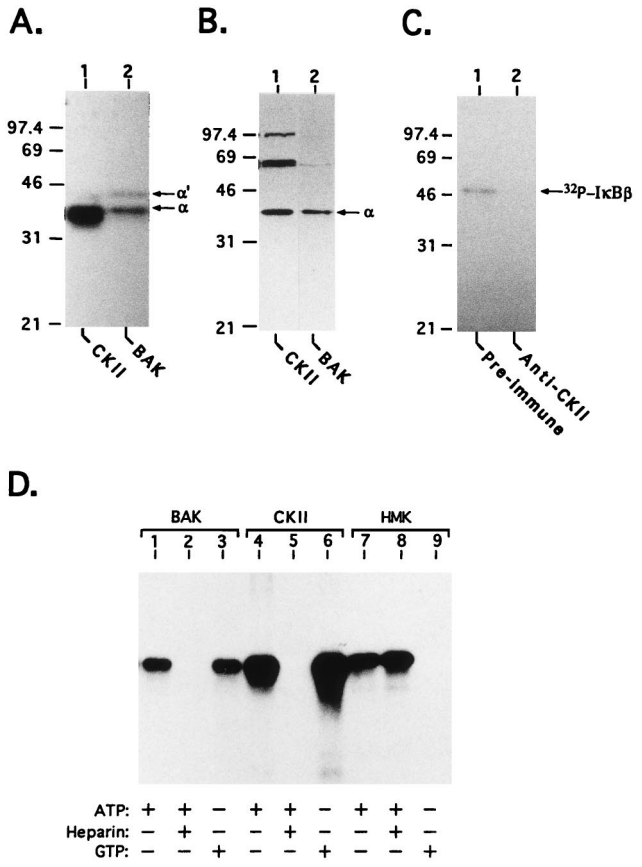


FIG. 5. Identification of BAK. (A) Electrophoretic properties of BAK. Whole-cell extracts from Jurkat T cells (10 mg) were incubated with rIκBβ and nickel chelate agarose beads. Bound proteins were resolved by SDS-PAGE in gels polymerized in the presence of purified rIκBβ (lane 2). Purified CKII was analyzed in lane 1. In-gel kinase assays were performed as described in Materials and Methods. Molecular mass standards are given in kilodaltons. Arrows indicate the positions of the α and α′ catalytic subunits of CKII. (B) Reactivity of BAK with anti-CKII antibodies. Affinity-purified BAK (lane 2) and CKII (lane 1) were fractionated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with anti-CKII antibodies. The immunoreactive 40-kDa species corresponding to the α subunit of CKII is indicated with an arrow. (C) Immunodepletion of CKII from BAK. Affinity purified BAK was incubated with either a normal rabbit serum (lane 1) or anti-CKII antibodies (lane 2) in the presence of protein A-agarose. Clarified supernatant proteins were used in kinase reactions supplemented with [γ-^{32}P]ATP and rIκBβ substrate. Phosphorylated products were resolved by SDS-PAGE and visualized by autoradiography. (D) Pharmacological properties of BAK. Kinase reactions (20 μl) containing rIκBβ as the substrate and either BAK (lanes 1 to 3), CKII (lanes 4 to 6), or HMK (lanes 7 to 9) were performed in the presence of the indicated combinations of [γ-^{32}P]ATP, [γ-^{32}P]GTP, and heparin. Radiolabeled products were resolved by SDS-PAGE and detected by autoradiography.

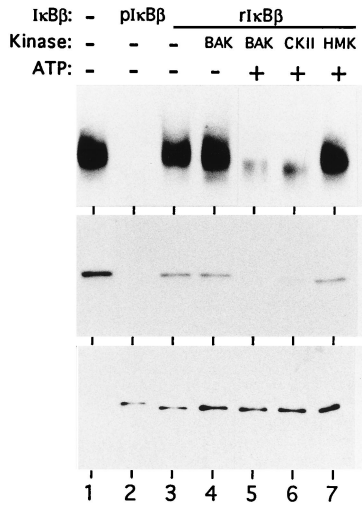


FIG. 6. BAK- and CKII-mediated phosphorylation of rIκBβ rescues its c-Rel inhibitory function. In vitro kinase reactions supplemented with the indicated kinase and rIκBβ substrate were carried out in the presence or absence of ATP (25 μM). Phosphorylated products were incubated with whole-cell extracts (5 μg) from A293T transfectants overexpressing c-Rel. Control reactions (lane 2) contained pIκBβ derived from transfected A293T cells. Resultant Rel-IκB complexes were added to DNA binding reactions containing radiolabeled κB-pd probe and were irradiated with UV light. Nucleoprotein complexes were analyzed by polyacrylamide gel electrophoresis under either nondenaturing (top panel) or denaturing (middle panel) conditions. To monitor modifications of IκBβ substrate, aliquots from each reaction mixture were fractionated on a separate SDS-polyacrylamide gel, transferred to a PVDF membrane, and immunoblotted with monoclonal anti-FLAG M2 antibody (bottommost panel).

phosphorylated with either BAK or CKII and then incubated with c-Rel homodimers from A293T cell transfectants. The resultant complexes were mixed with photoreactive κB oligonucleotides, irradiated with UV light, and then fractionated on polyacrylamide gels under either native (Fig. 6, top panel) or denaturing (middle panel) conditions. As expected, basally phosphorylated IκBβ derived from A293T cell transfectants prevented the formation of c-Rel-specific nucleoprotein complexes (lane 2), whereas rIκBβ was defective (lane 3). Strikingly, phosphorylation of rIκBβ with either affinity-purified BAK or CKII reconstituted its inhibitory function, as evidenced by the loss of nucleoprotein complexes containing c-Rel (lanes 5 and 6). However, incubation of rIκBβ with BAK in ATP-depleted reaction mixtures failed to rescue IκBβ function (lane 4), thus excluding the possibility that the BAK preparations were contaminated with IκB proteins. Furthermore, control phosphorylation reactions performed with HMK, which modifies the N-terminal tag present in rIκBβ, had no effect on this IκBβ function. These findings suggest that CKII-mediated phosphorylation of IκBβ is required to endow this inhibitor with the capacity to antagonize the DNA binding activity of c-Rel.

The PEST domain of IκBβ contains sites for basal phosphorylation. Having established that CKII regulates the c-Rel inhibitory function of IκBβ in vitro, we next performed experiments to identify candidates for the relevant phosphoacceptor sites. In initial studies, purified rIκBβ was phosphorylated with CKII, digested with endoproteinase Lys-C, and fractionated on high-resolution Tris-Tricine gels. As shown in Fig. 7A, a major phosphopeptide of approximately 10 kDa was liberated from the full-length inhibitor by Lys-C cleavage (lanes 1 and 2). Similar results were obtained in parallel experiments using pIκBβ isolated from A293T transfectants that were metabolically radiolabeled with $^{32}P_i$ (lanes 5 and 6). The 10-kDa phosphopeptide identified in these experiments failed to react with an antiserum directed against the N-terminal region of IκBβ (amino acids 21 to 40). However, this proteolytic product retained the capacity to bind a nickel chelate resin (lanes 4 and 8), thus confirming its origin from the HIS-tagged, C-terminal PEST domain of IκBβ.

To delineate further the affected region of the IκBβ PEST domain, expression vectors encoding truncated forms of the IκBβ protein were transiently transfected into Jurkat T lymphocytes. After metabolic labeling with $^{32}P_i$, these IκBβ proteins were immunopurified from recipient cell lysates and analyzed for levels of radioisotope incorporation. As shown in Fig. 7B, wild-type IκBβ [IκBβ(WT)] (lane 1) and an N-terminal deletion mutant lacking amino acids 1 to 56 [IκBβ(57-356)] (lane 2) were ^{32}P labeled in vivo with comparable efficiencies when normalized for relative expression levels (lanes 5 and 6). These data are consistent with the peptide mapping results (Fig. 7A) and suggest that the bulk of the basal phosphoryla-

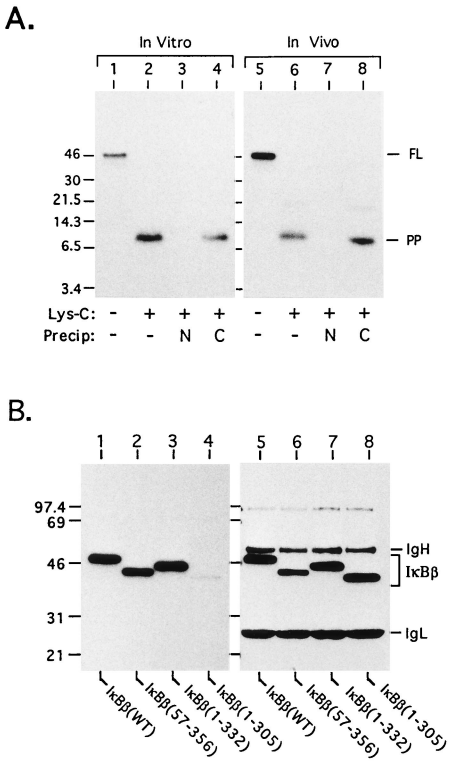


FIG. 7. Localization of basal phosphorylation sites in the PEST domain of IκBβ. (A) CKII phosphorylates IκBβ within the C-terminal PEST domain. Purified rIκBβ was in vitro phosphorylated with CKII in the presence of [γ-^{32}P] ATP (lane 1). Phosphorylated IκBβ from metabolically radiolabeled A293T transfectants was purified by immunoprecipitation with anti-FLAG M2 antibody (lane 5). Radiolabeled IκBβ protein was digested with endoproteinase Lys-C, and the cleavage products were resolved by high-resolution SDS-PAGE (lanes 2 and 6). Alternatively, prior to electrophoresis Lys-C-derived phosphopeptides were precipitated (Precip) with antisera specific for the N terminus of IκBβ (N [lanes 3 and 7]) or a nickel chelate resin (C [lanes 4 and 8]). Phosphorylated species corresponding to full-length IκBβ (FL) and the major Lys-C cleavage product (PP) are indicated. Similar results were obtained in peptide mapping experiments conducted with BAK-phosphorylated IκBβ (data not shown). (B) Deletion of the IκBβ PEST domain prevents basal phosphorylation in vivo. FLAG-tagged forms of wild-type IκBβ or the indicated deletion mutants were overexpressed in transfected Jurkat T cells, metabolically labeled with $^{32}P_i$, and subjected to immunoprecipitation with anti-FLAG M2 antibody. Immunopurified IκBβ was resolved by SDS-PAGE, transferred to a PVDF membrane, and subjected sequentially to autoradiography (lanes 1 to 4) and immunoblotting with anti-FLAG M2 antibody (lanes 5 to 8). IgH and IgL, immunoglobulin heavy and light chains, respectively.

tion sites in IκBβ reside downstream of position 56. Furthermore, an IκBβ mutant terminated in the PEST region at His-332 [IκBβ(1-332)] (lane 3) was phosphorylated to an extent similar to that of wild-type IκBβ. However, the deletion of 27 additional amino acids from the C-terminal end of IκBβ(1-332) led to a dramatic reduction in basal phosphorylation [IκBβ(1-305)] (lane 4). In contrast to the striking differences in their basal phosphorylation status, the steady-state levels of mutants IκBβ(1-332) and IκBβ(1-305) were indistinguishable (lanes 7 and 8). Coupled with the peptide mapping studies (Fig. 7A), these data suggest that the major sites for basal phosphorylation in the PEST domain of IκBβ reside between amino acids 306 and 332.

Identification of putative phosphoacceptor sites in the IκBβ PEST domain. As illustrated in Fig. 8A, the PEST sequences located between amino acids 306 and 332 of human IκBβ (45) contain six serine residues, two of which (Ser-313 and Ser-315) reside within consensus CKII phosphorylation sites ([S/T]-X-X-[E/D]). These CKII sites are conserved in the murine homolog of IκBβ (72). Of note, phosphoserine can substitute for the acidic residue in the fourth position of this motif (40), thus creating a potential secondary site for CKII-mediated phosphorylation at Ser-312 in IκBβ if Ser-315 is appropriately modified. This region of the IκBβ PEST domain is devoid of threonine residues. To determine whether full-length IκBβ is phosphorylated exclusively on serines, we isolated radiolabeled IκBβ from Jurkat T cell transfectants that were cultured in media containing $^{32}P_i$. As shown in Fig. 8B (left-hand panel), phosphoamino acid analysis of basally modified IκBβ from recipient cell extracts revealed the presence of phosphoserine but neither phosphothreonine nor phosphotyrosine moieties. A similar pattern of serine-specific phosphorylation was observed in parallel studies with rIκBβ when subjected to in vitro phosphorylation with BAK (Fig. 8B, right-hand panel). These results are fully consistent with the hypothesis that serine residues positioned within the PEST domain of IκBβ are basally phosphorylated.

To extend these findings, the cDNA encoding full-length IκBβ was modified by site-directed mutagenesis to replace Ser-313 and Ser-315 with alanine, thus simultaneously disrupting both the primary and secondary consensus CKII sites. The resultant mutant, termed S313/315A, was first expressed as an rIκBβ protein in *E. coli* and then tested as a substrate for phosphorylation by affinity-purified BAK. As shown in Fig. 8C, mutations at Ser-313 and Ser-315 in IκBβ (lane 2) reduced its in vitro labeling efficiency by at least 95% relative to that of the wild-type inhibitor (lane 1). Furthermore, in vivo radiolabeling experiments conducted with this double point mutant revealed a 70% ± 1% diminution (based on three separate experiments) in the overall magnitude of phosphorylation compared with that of the wild-type control (Fig. 8C, lanes 5 and 6). Parallel immunoblotting studies indicated that these IκBβ substrates were expressed at comparable steady-state levels in vivo (lanes 7 and 8), suggesting that the S313/S315A mutation does not significantly alter IκBβ stability. Coupled with the results shown in Fig. 7B (lane 4), these quantitative differences between the in vitro and in vivo phosphorylation patterns of IκBβ raise the possibility that the endogenous inhibitor is modified at serine residues in addition to Ser-313 and Ser-315. The locations of these additional phosphoacceptor sites remain unknown. Notwithstanding this uncertainty, these data with site-directed mutants suggest that phosphoryl group transfer to Ser-313 and/or Ser-315 of IκBβ contributes significantly to its basal phosphorylation state.

Ser-313 and Ser-315 of IκBβ regulate its functional interaction with c-Rel. Studies presented in Fig. 1 and 2 firmly established that basal phosphorylation of IκBβ is required to inhibit c-Rel but not RelA DNA binding activity. To determine whether site-specific phosphorylation at Ser-313 and Ser-315 is involved in this posttranslational targeting mechanism, we

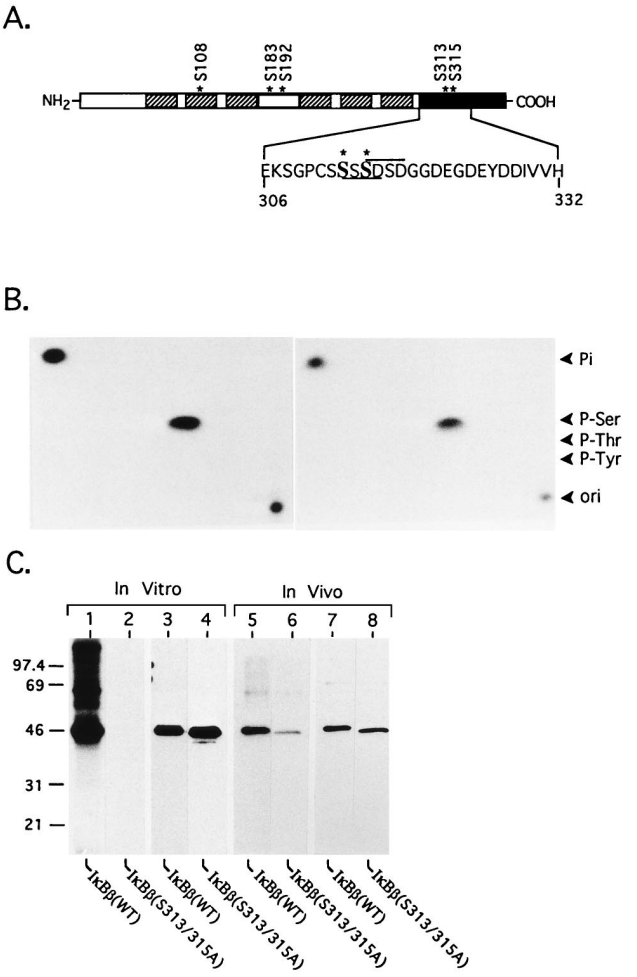
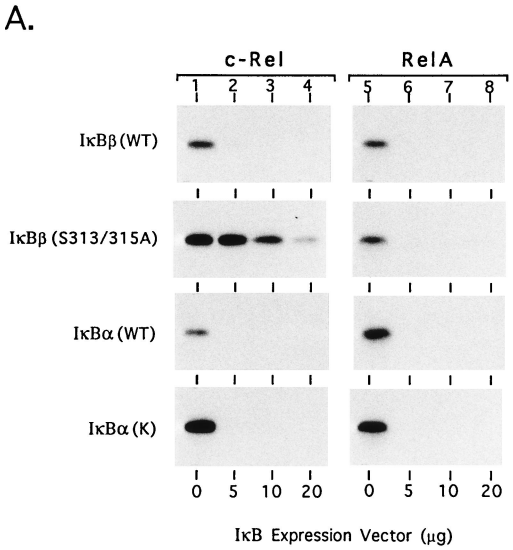


FIG. 8. Identification of Ser-313 and Ser-315 as putative phosphoacceptor sites in IκBβ. (A) The PEST domain of IκBβ contains two CKII consensus sites. The schematic representation of human IκBβ shown depicts the N-terminal signal response domain (N-terminal open box) (amino acids 1 to 56), a central domain composed of six ankyrin repeat motifs (hatched boxes) (amino acids 57 to 305), and a highly acidic C-terminal domain containing PEST-like sequences (closed box) (amino acids 306 to 356). Potential CKII phosphorylation sites, two of which are located in the PEST domain, are indicated (asterisks). Sequences implicated in basal phosphorylation of the PEST domain (amino acids 306 to 332) (Fig. 7) are shown. (B) IκBβ is basally phosphorylated at serine residues in vivo (left-hand panel) and in vitro (right-hand panel). Jurkat T cells were transfected with an IκBβ expression vector and metabolically labeled with $^{32}P_i$. Ectopic IκBβ was sequentially immunoprecipitated from cytoplasmic extracts with IκBβ-specific (C-20) and anti-FLAG M2 antibodies. Recombinant IκBβ was phosphorylated in vitro with BAK and [γ-^{32}P]ATP, and this was followed by the same immunoprecipitation procedure. Phosphorylated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and subjected to phosphoamino acid analysis in parallel with phosphoamino acid standards (phosphorylated Ser, Thr, and Tyr [P-Ser, P-Thr, and P-Tyr, respectively]). The positions of free phosphate (Pi) and the sample origin (ori) are indicated. (C) Phosphorylation patterns of site-directed IκBβ mutants. Bacterially expressed IκBβ [IκBβ(WT)] or a mutant containing alanine substitutions at Ser-313 and Ser-315 [IκBβ(S313/315A)] was coupled to anti-FLAG M2 agarose beads and phosphorylated in vitro with BAK in the presence of [γ-^{32}P]ATP. Alternatively, transfected Jurkat T lymphocytes were metabolically labeled with $^{32}P_i$ and ectopic IκBβ was fractionated from whole-cell extracts by immunoprecipitation with anti-FLAG M2 antibody. Products were resolved by SDS-PAGE, transferred to a PVDF membrane, and detected by sequential autoradiography (lanes 1, 2, 5, and 6) and immunoblotting with anti-FLAG M2 antibody (lanes 3, 4, 7, and 8).

tested whether mutants of IκBβ lacking these sites form latent complexes with Rel homodimers in vivo. For these studies, Jurkat T lymphocytes were transiently cotransfected with Rel expression vectors and graded amounts of plasmids encoding either wild-type IκBβ or the double mutant S313/315A. For comparisons, we also prepared transfectants expressing either wild-type IκBα or a mutant containing alanine substitutions at each of the potential CKII phosphorylation sites in the IκBα PEST domain (mutant K [25]). Nuclear extracts from these transfectants were prepared and analyzed in DNA-protein cross-linking assays.

As shown in Fig. 9A, the DNA binding activity of nuclear c-Rel homodimers (lane 1) was efficiently titrated by wild-type IκBβ in a dose-dependent fashion (lanes 2 to 4). Consistent with this, c-Rel complexes containing this ectopic inhibitor were readily detected in the cytoplasmic compartment (data not shown). In sharp contrast, despite comparable levels of steady-state expression (Fig. 8C), in vivo titration experiments performed with the S313/315A mutant of IκBβ revealed a profound defect in its c-Rel inhibitory activity (Fig. 9A, second panel from top, lanes 2 to 4). Furthermore, the simultaneous disruption of Ser-313 and Ser-315 had no detectable effect on the capacity of IκBβ to block the nuclear activity of RelA homodimers (lanes 6 to 8), suggesting that the regulatory potentials of these two serines are Rel subunit specific. In keeping with results shown in Fig. 2C and 4, mutants of IκBα that escape from basal phosphorylation in vivo (46, 48, 64) were fully competent to inhibit either c-Rel or RelA DNA binding (Fig. 9A, bottom panels). Of note, we observed partial inhibition of c-Rel DNA binding activity in parallel studies conducted with IκBβ proteins containing single point mutations at either Ser-313 or Ser-315 (data not shown). These results suggest that phosphorylation at both serines in IκBβ is required to generate the fully functional inhibitor.

Prior studies have established that the C terminus of human c-Rel contains a transactivation domain that stimulates the expression of certain genes under NF-κB control (6, 23). To extend our biochemical findings, we examined the ability of IκBβ to inhibit c-Rel-directed transcription in vivo. For these studies, A293T cells were cotransfected with a c-Rel expression vector, a CAT reporter construct under the transcriptional control of two NF-κB/Rel binding sites from the HIV enhancer (HIV-κB-CAT), and graded amounts of effector plasmids encoding either wild-type IκBβ or the S313A/S315A mutant. As shown in Fig. 9B, wild-type IκBβ inhibited c-Rel-dependent transcription of the CAT reporter gene in a dose-dependent manner. In keeping with the biochemical results, these functional studies revealed a profound defect in the ability of the S313A/S315A mutant to terminate the transactivating function of c-Rel. Taken together, these findings strongly suggest that basal phosphorylation of IκBβ at Ser-313 and Ser-315 is required to endow this inhibitor with its c-Rel targeting function in vivo.



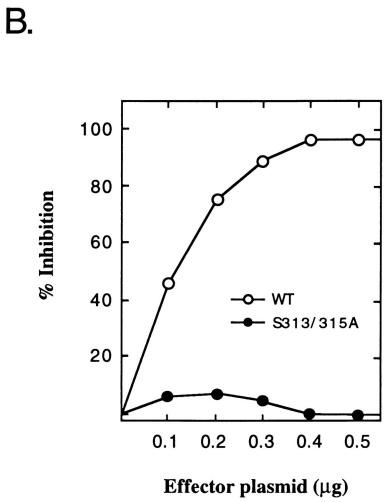


FIG. 9. Mutations at Ser-313 and Ser-315 in IκBβ disrupt its ability to inhibit c-Rel function. (A) Regulation of nuclear c-Rel activity. Jurkat T cells were transfected with expression vectors encoding either c-Rel (15 μg) (lanes 1 to 4) or RelA (10 μg) (lanes 5 to 8) along with graded doses (0 to 20 μg) of the indicated FLAG-tagged IκB constructs. Nuclear extracts from recipient cells (5 μg) were incubated with a photoreactive κB-pd probe and irradiated with UV light. c-Rel or RelA homodimer-DNA adducts were immunoprecipitated with subunit-specific antisera after immunodepletion of other minor Rel-DNA complexes. Adducts were fractionated by SDS-PAGE and visualized by autoradiography. (B) Effect of site-directed mutations in IκBβ on c-Rel-directed transcription. A293T cells were cotransfected with a c-Rel expression vector (2 μg), increasing amounts of the indicated IκBβ expression vector, and HIV-κB-CAT (0.5 μg). Total input DNA was normalized for all transfections by the addition of empty pCMV4 vector. After 48 h in culture, whole-cell extracts were prepared, normalized for total protein, and assayed for CAT activity. Results from three independent transfections are reported as the mean percent inhibition of CAT activity in cells expressing IκB effector versus those expressing c-Rel alone (fold induction, 7.1 ± 0.5). WT, wild type.

DISCUSSION

Members of the IκB family of proteins regulate the inducible activity of NF-κB/Rel complexes from the cytoplasmic compartment (9, 74). Two of the major constituents of this inhibitor family, termed IκBα and IκBβ, serve as targets for receptor-mediated signals that culminate in the rapid translocation of NF-κB/Rel proteins to their nuclear site of action. Emerging evidence suggests that both of these inhibitors are subject to degradation by the proteasome pathway in response to a variety of agents that activate NF-κB (2, 18, 21, 49, 54, 72). Although IκBα and IκBβ likely interact with the same set of NF-κB/Rel family members, it appears that these two inhibitors display distinct responses to different inducers of NF-κB activity (72). Furthermore, the gene encoding IκBα is under the control of NF-κB (34, 44), whereas the expression of the IκBβ transcription unit is regulated by an NF-κB-independent mechanism (49, 72). The differential regulation of these IκB proteins at the level of transcription correlates with the unique ability of IκBα to downregulate the nuclear activity of NF-κB in vivo (10).

The present study highlights yet another important functional distinction between IκBα and IκBβ involving their c-Rel targeting mechanisms. Specifically, we have assembled several lines of evidence suggesting that basal phosphorylation of IκBβ but not IκBα is required for stable formation of latent c-Rel complexes. First, bacterially expressed forms of IκBβ fail to associate with c-Rel homodimers, whereas unmodified IκBα is fully competent to execute this function (Fig. 3). Second, in vitro phosphorylation of bacterial IκBβ by BAK derived from unstimulated Jurkat T cells rescues its defect in c-Rel targeting (Fig. 6). Third, latent c-Rel complexes containing basally phosphorylated IκBβ but not IκBα are efficiently converted to active DNA binding proteins by phosphatase treatment (Fig. 4). Thus, in sharp contrast to the behavior of IκBα, the functional interaction of IκBβ with c-Rel is regulated by basal phosphorylation.

Despite this difference, IκBα and IκBβ both contain PEST sequences in their C-terminal regions, a property which is characteristic of many short-lived proteins (56, 57). In the case of IκBα, the PEST domain appears to regulate basal turnover of this inhibitor by a mechanism involving phosphorylation at serine and threonine residues (46, 48, 64). In contrast to the data presented here (Fig. 2C), it has been reported that phosphorylation of the PEST domain of avian IκBα is required for efficient inhibition of c-Rel DNA binding activity in vitro (59). Although the basis for this apparent discrepancy regarding IκBα remains unclear, our studies demonstrate that basal phosphorylation of the PEST domain of human IκBβ plays an important role in the acquisition of its c-Rel inhibitory function. In particular, we have found that simultaneous disruption of two potential phosphorylation sites located in the PEST domain of human IκBβ (Ser-313 and Ser-315) leads to a significant reduction in basal phosphorylation (Fig. 8C) and c-Rel binding (~20% that of the wild type [data not shown]). More importantly, mutations introduced at both serines severely impaired the c-Rel inhibitory function of IκBβ when expressed in vivo (Fig. 9). This function was only partially restored in experiments conducted with single point mutants, thus suggesting a requirement for phosphorylation at both serines to generate the fully functional inhibitor (data not shown).

These observations indicate that Ser-313 and Ser-315 of IκBβ play a significant role in the efficient formation of IκBβ–c-Rel complexes, presumably as essential phosphoacceptor sites. We cannot formally exclude the possibilities that perturbation of these regulatory serines indirectly affects the overall structural integrity of IκBβ or influences substrate recognition by other kinases involved in the basal phosphorylation of IκBβ. We consider the first possibility to be unlikely, because mutants of IκBβ lacking Ser-313 and Ser-315 retain the capacity to regulate RelA in vivo (Fig. 9A). However, on the basis of metabolic radiolabeling studies with site-directed mutants, residues in IκBβ other than Ser-313 and Ser-315 are subject to basal phosphorylation in transfected T cells (Fig. 8C). Like Ser-313 and Ser-315, these additional sites appear to reside in the C-terminal region of IκBβ (Fig. 7). As such, it is conceivable that the phosphorylation state of Ser-313 and Ser-315 influences phosphoryl group transfer to these undefined sites.

Prior studies have suggested that the serine/threonine kinase CKII mediates the basal phosphorylation of the PEST domain present in IκBα, which modestly affects its basal turnover rate (8, 46, 48, 64). The data presented here indicate that CKII also phosphorylates the PEST domain of IκBβ, albeit with distinct functional consequences. Specifically, we have partially purified an IκBβ kinase from unstimulated Jurkat T cells by exploiting its in vitro capacity to physically associate with unphosphorylated IκBβ. This kinase, termed BAK, is immunoreactive with CKII-specific antibodies, comigrates with the catalytic subunits of CKII in SDS-polyacrylamide gels, and exhibits pharmacological properties similar to those of CKII (Fig. 5). On the basis of in vitro phosphorylation assays, both CKII and BAK can rescue the c-Rel targeting defect exhibited by unmodified IκBβ (Fig. 6). Furthermore, affinity-purified BAK fails to efficiently phosphorylate IκBβ proteins lacking Ser-313 and Ser-315. These potential phosphorylation sites reside within two CKII consensus sites and are required for efficient targeting of IκBβ to c-Rel in vivo (Fig. 9). Taken together, these experimental results link BAK and CKII in terms of their IκBβ recognition specificities and functional effects on the c-Rel inhibitory activity of IκBβ. Although these correlative findings do not constitute proof that CKII is the physiological IκBβ kinase responsible for mediating IκBβ–c-Rel interactions, it seems likely that the phosphorylation site specificity of the bona fide effector overlaps that of CKII.

In summary, our biochemical studies strongly suggest that IκBβ must be basally phosphorylated at Ser-313 and Ser-315, either by CKII or a closely related kinase, in order to endow this inhibitor with its c-Rel targeting function. These results firmly establish that phosphorylation of an IκB protein can facilitate not only signal-dependent breakdown (2, 18) but also stable formation of a latent IκB–Rel complex. We have also assembled in vitro evidence that this targeting mechanism is subunit specific, a finding which could have important implications for the differential regulation of individual NF-κB/Rel family members. For example, regulation of IκBβ at the level of phosphorylation may significantly affect its functional interplay with select Rel dimers. In keeping with this proposal, recent studies have identified an unphosphorylated form of IκBβ that accumulates in cells under certain stimulatory conditions (69). Unlike basally phosphorylated IκBβ, which retains NF-κB in the cytoplasm, its unphosphorylated counterpart appears to form complexes with NF-κB that can enter the nucleus (69). Coupled with the present study, these observations raise the intriguing possibility that regulation of IκBβ phosphorylation at Ser-313 and Ser-315 by the appropriate protein kinase/phosphatase system represents a central mechanism to help achieve subunit-specific control of Rel-directed transcription.

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